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Composition Studies on Tobacco II. Isolation and Identification of Stigmasterol from Flue-cured Leaves

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Introduction

As indicated in Part I of this series (Dymicky and Stedman, 1958), the occurrence of steroidal substances in tobacco has been known for over forty years. Only recently, however, has work on the identification of specific compounds of this type been forthcoming. Prior to the present series of papers two steroidal compounds isolated from tobacco had been characterized: τ-sitosteryl D-glucoside from chewing tobacco (Khanolkar *et al.*, 1955), and stigmasterol from blended cigarette smoke (Kosak *et al.*, 1957). Part I of the current series added β-sitosteryl D-glucoside from flue-cured tobacco leaves to this list. The present report concerns the isolation of stigmasterol from the same source.

Experimental and Results

A flow sheet of the separation scheme is given in **figure 1.** The details of the fractionation are as follows: thirty-seven kg. of Type 12 tobacco (mixed U. S. grades) were extracted under reflux with 320 l. of Skellysolve B. The extract was concentrated to eight l. and a one l. aliquot of this evaporated *in vacuo* to a dark-brown, waxy residue which was thoroughly washed with 12 per cent hydrochloric acid and water to remove bases. The residue was dissolved in petroleum ether, and the solution was extracted successively with 620 ml of 60 per cent, 800 ml of 80 per cent and 900 ml of 90 per cent aqueous methanol to remove xanthophylls and other materials. The petroleum ether solution was evaporated to dryness and exeracted with 700 ml acetone. The acetone extract was evaporated to dryness, the residue dissolved in three l. petroleum ether and the later chromatographed on one lb. of activated Fisher alumina[2] in a 1.5 in. diameter column. After elution of some hydrocarbons and other substances with petroleum ether and benzene (petroleum ether, petroleum ether containing 20 per cent and 50 per cent benzene, benzene in succession), a 1:1 mixture of benzene-diethyl ether eluted a fraction which, on evaporation of the solvent and trituration with ethanol, yielded 80 mg of crude sterol. Two recrystallizations from 95 per cent ethanol gave stigmasterol, m.p. 162°-164°C, $[\alpha]^{20}_D$ −47.4° (chloroform) (lit., stigmasterol: m.p. 168°-169°C, $[\alpha]^{20}_D$ −47.3° in chloroform (Ott and Ball, 1944); m.p. 170°C, $[\alpha]^{20}_D$ −45.1° in chloroform (Stoll and

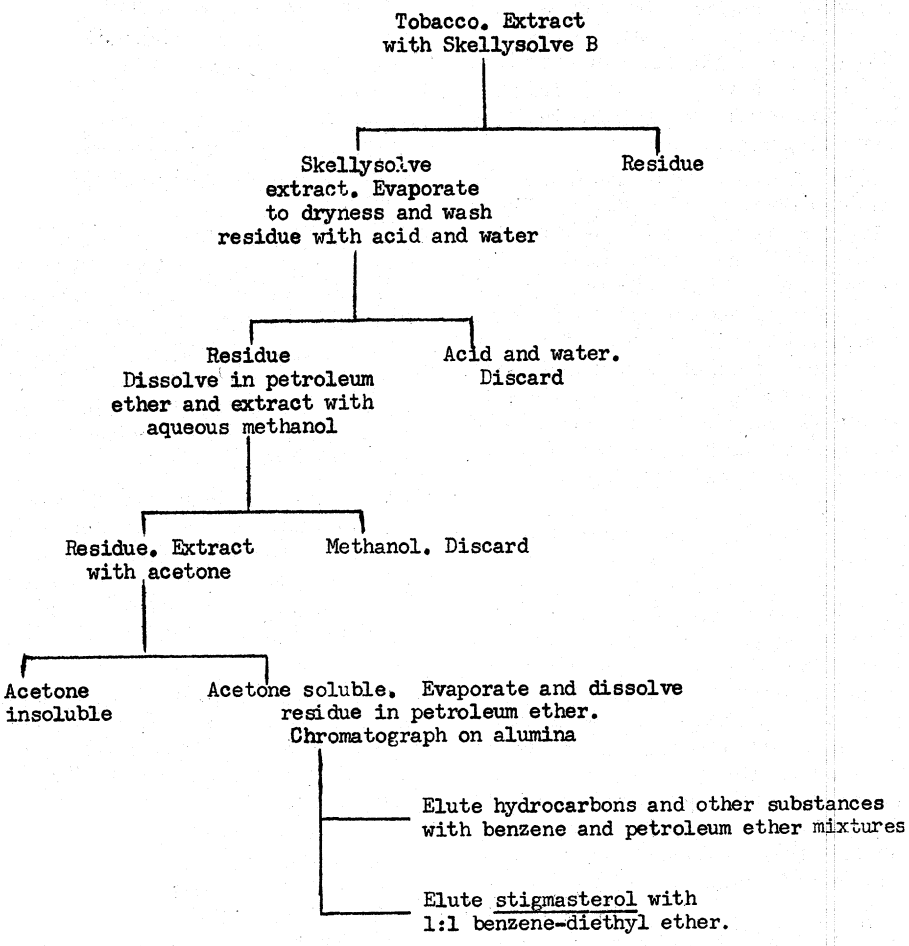

Figure 1. Isolation of stigmasterol from flue-cured tobacco leaves.

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[2] *Mention of any commercial product does not constitute endorsement of the product by the Department of Agriculture over others of a similar nature.*

Jucker, 1955); m.p. 167°C, $[\alpha]^{20}_D$ −49° in chloroform (Turnbull, 1950).

The infrared spectrum of the compound was identical with that of authentic stigmasterol, including the characteristic absorption bands at 1051, 1023, 1007, 972, 960, 925, 838 and 799 cm^{-1}. The strong band for a Δ^{22} *trans* configuration reported to occur at 970 cm^{-1} in the case of stigmasterol (Turnbull, 1950) was found at 972 cm^{-1} in the isolated compound.

Twenty mg. of the isolated sterol was then treated for 24 hours at room temperature with a mixture of one ml of benzoyl chloride and five ml of anhydrous pyridine. The mixture was poured into cold water and the solution extracted with ether. The ethereal solution was washed successively with 10 per cent HCl solution, water, five per cent sodium bisulfate solution and water and dried over sodium sulfate. Evaporation of the ether and recrystallization of the product from methanol gave a compound, m.p. 157°-159°C, $[\alpha]_D$ −24.7° in chloroform (lit., stigmasteryl benzoate: m.p. 160.5-161.5°C, $[\alpha]_D$ −24.5° in chloroform (Ott and Ball, 1950); m.p. 160°C (Stoll and Jucker, 1955)). Infrared spectroscopy showed the strong absorption bands at 1720 and 1275 cm^{-1} characteristic of benzoates.

Discussion

Phytosterols from plant material are frequently isolated as mixtures of closely related compounds. Separation of the components of such mixtures is quite difficult (Stoll and Jucker, 1955; Bergmann, 1953). The melting point of the stigmasterol reported above was low compared to literature values, and the isolated material may have contained small amounts of other phytosterols. However, the optical rotation of the free sterol, which is a more reliable criterion for identification in such cases, compares well with the values in the literature. Other compounds which might be expected to show an infrared spectrum similar to stigmasterol are the C_{24} homologues or epimers, brassicasterol (24b-methyl-), and poriferasterol (24a-ethyl-). The melting points and optical rotations of these related compounds and derivatives thereof are significantly different from those reported above for the isolated stigmasterol.

In reporting the presence of stigmasterol in cigarette smoke, Kosak *et al.* cited the possibility that the sterol might have been present originally as a glycoside and that the acid-washing step in the isolation procedure might have caused hydrolysis of the sterolin. Although the monoglucoside of stigmasterol has been synthesized (Gisvold, 1934), examination of the literature has failed to reveal a clearcut isolation study in which a glycoside of stigmasterol has been characterized. Clewer *et al.* (1915) have isolated a compound which showed the precipitation at the ether-aqueous acid interface characteristic of phytosterolins, but on hydrolysis, a mixture of stigmasterol and "sitosterol" was obtained. The glucosides isolated by Thornton *et al.* (1940) also gave a mixture of sterols on hydrolysis although stigmasterol was present in a concentration of 24 per cent. In contrast, numerous reports on the isolation of free or esterified stigmasterol exist[3], including the works of Clewer *et al.* and Thornton *et al.* cited above.

In connection with the question of bound stigmasterol, current work in this laboratory has shown the presence in tobacco of a steroidal substance of high melting point which exhibits the characteristics of a glycoside and shows absorption bands at 960 and 972 cm^{-1} similar to stigmasterol. Another substance isolated here shows certain physical properties similar to the aglycone obtained from the above on acid hydrolysis, but dissimilar to stigmasterol in some respects.

The current study confirms the implications drawn in Part I of this series: at least part of the steroidal content of smoke from blended cigarettes may be contributed by the flue-cured tobacco in the blend. Furthermore, the stigmasterol found in the smoke probably originates from simple volatilization of tobacco constituents and not from sources such as selective dehydrogenation of sitosteryl glycosides of tobacco during heating.

The quantity of stigmasterol found in tobacco in the present study is small compared to the weight of tobacco extracted. This is not unexpected since no attempt at quantitative isolation and extraction was made. In view of the potential importance of stigmasterol as a starting material for the synthesis of steroids having pharmacological activity it would appear worthwhile to determine the level of this and other phytosterols in tobacco. This problem is now under investigation and will be reported at another time.

[3] *In many cases, initial saponification of the plant extract was performed routinely, making it impossible to distinguish free and bound sterol. Saponification does not hydrolyze sterol glycosides.*

Summary

Stigmasterol has been isolated and identified as a constituent of American flue-cured tobacco leaves. When related to another published report on stigmasterol in blended cigarette smoke, the present findings would indicate that at least part of the sterols in smoke may arise from the flue-cured tobacco in the blend. The possibility of stigmasterol occurring in glycosidated form is discussed.

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